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Anti-inflammatory, anti-bacterial and anti-acetylcholinesterase activities of two isoquinoline alkaloids-scoulerine and cheilanthifoline

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Abstract

Corydalis plants containing isoquinoline alkaloids are reported to possess promising pharmacological properties for the treatment of important diseases including cancer, inflammation, Alzheimer's disease and microbial infections. As part of a wider program investigating Bhutanese medicinal plants, we have previously identified eight isoquinoline alkaloids from C. dubia. Out of these, we report here on two of the major alkaloids, scoulerine (1) and cheilanthifoline (2) and their inhibitory activities against acetylcholinesterase (anti-AChE), tumor necrosis factor alpha (anti TNF-α) and a bacterial strain, *Helicobacter pylor*i. Both alkaloids showed weak anti TNF-α and antibacterial activities. However, the anti-AChE activity of scoulerine (1) was promising as it significantly inhibited AChE with a minimum inhibitory requirement (MIR) value of 0.0015 nmol, which was two-fold better than the reference drug, galanthamine (MIR value of 0.003 nmol). As there are limited anti-Alzheimer's chemotherapeutics, scoulerine (1) is worthy of further exploration, including lead optimization, structure-activity-relationship studies, analog development, pharmacodynamics and *in vivo* animal studies.

Keywords

alkaloids-scoulerine, activities, anti-acetylcholinesterase, anti-inflammatory, cheilanthifoline, isoquinoline, anti-bacterial, two

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Anti-inflammatory, Anti-bacterial and Anti-acetylcholinesterase Activities of two Isoquinoline Alkaloids–Scoulerine and Cheilanthifoline

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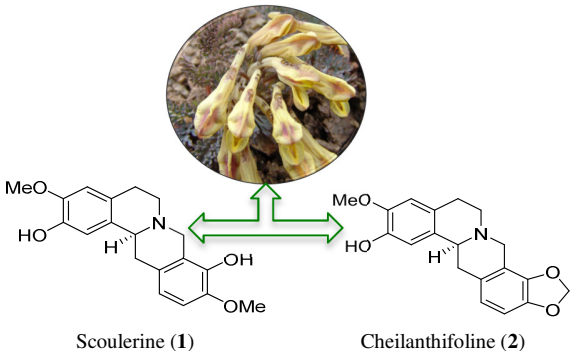
Corydalis plants containing isoquinoline alkaloids are reported to possess promising pharmacological properties for the treatment of important diseases including cancer, inflammation, Alzheimer's disease and microbial infections. As part of a wider program investigating Bhutanese medicinal plants, we have previously identified eight isoquinoline alkaloids from *C. dubia*. Out of these, we report here on two of the major alkaloids, scoulerine (**1**) and cheilanthifoline (**2**) and their inhibitory activities against acetylcholinesterase (anti-AChE), tumor necrosis factor alpha (anti TNF-α) and a bacterial strain, *Helicobacter pylori*. Both alkaloids showed weak anti TNF-α and antibacterial activities. However, the anti-AChE activity of scoulerine (**1**) was promising as it significantly inhibited AChE with a minimum inhibitory requirement (MIR) value of 0.0015 nmol, which was two-fold better than the reference drug, galanthamine (MIR value of 0.003 nmol). As there are limited anti-Alzheimer's chemotherapeutics, scoulerine (**1**) is worthy of further exploration, including lead optimization, structure-activity-relationship studies, analog development, pharmacodynamics and *in vivo* animal studies.

Keywords: Acetylcholinesterase inhibitor, Isoquinoline alkaloid, Anti-inflammatory, Antimicrobial, Medicinal plant, *Corydalis dubia*.

Historically, natural products have played an important role in traditional medicines and drug discoveries. Drugs discovered from natural sources, especially medicinal plants, continue to provide new drug lead compounds and also enter clinical trials, particularly as antimalarial, anticancer and antimicrobial agents. A detailed analysis of new medicines approved by the US Food and Drug Administration (FDA) between 1981 and 2010 revealed that 34% of those medicines that were based on small molecules were natural products or direct derivatives of natural products [1-2]. Medicinal plants belonging to the genus *Corydalis* (Family Papaveraceae) are known to contain drug-like bioactive isoquinoline alkaloids with promise for treating inflammation, microbial infections, cancers, and Alzheimer's disease (AD) [3].

As part of a wider program investigating Bhutanese medicinal plants, we have previously carried out phytochemical and biological activity studies of the three Bhutanese *Corydalis* species (*C. calliantha, C. crispa* and *C. dubia*), which resulted in the identification of number of isoquinoline alkaloids with potent antimalarial activity against the multidrug resistant *Plasmodium falciparum* strain [4-7]. The crude MeOH and $CHCl_3$ extracts of *C. dubia* showed anti-inflammatory activity with 58% and 62% inhibition of TNF-α production, respectively, in LPS activated THP-1 cells [8]. Traditionally, this medicinal plant is indicated for treating impure blood (detox), fever arising from infections of the liver, heart, lung, pancreas and kidney, and in alleviating neuralgia and complicated disorders (combination of defective air, bile and phlegm) that bore relevance to the symptoms of inflammation, microbial infections and AD [4]. Considering these, we have further investigated two major isoquinoline alkaloids - scoulerine (**1**) and cheilanthifoline (**2**) (Figure 1) for their anti-inflammatory, anti-bacterial and the anti-acetylcholinesterase (anti-AChE) activities. These two alkaloids are related since they are both biosynthesized in plants from tyrosine [9]. Protopine, one of the major alkaloids of this plant, is biosynthetically related to these two alkaloids and has shown broad-spectrum bioactivities, especially anti-inflammatory and AChE inhibitory activities [6,10-11].



Scoulerine (**1**) Cheilanthifoline (**2**)

Figure 1: Structure of the two major isoquinoline alkaloids (isolated from *C. dubia*), which were tested for their anti-inflammatory, anti-bacterial and anti-acetylcholinesterase activities.

Our studies revealed that scoulerine (**1**) and cheilanthifoline (**2**) also possess broad-spectrum biological activities. Both these alkaloids exhibited weak antibacterial and anti-inflammatory properties, but showed strong –moderate anti-acetylcholinesterase activity (Table 1). Plant derivatives are clinically known for their anti-inflammatory properties [12]. Out of 171 alkaloids isolated between 1907 and 2000, 137 were reported to have anti-inflammatory properties [13].

Table 1: Anti-AChE, anti-TNF-α and antibacterial activities of compounds **1** and **2** from *C. dubia*.

Compds/positive controls	Anti-AChE*	Anti-TNF-α**		Antimicrobial***	
	MIR (nmol)	TNF-α (pg/mL)	% inhibition	*H. pylori* MIZ (mm)	MIC (µg/mL)
MeOH extract	0.0015	566 ± 17	– (9)	6	2000
1					
2	0.31	541 ± 33	– (13)	5	250
Galanthamine[a]	0.003				
Dexamethasone[b]		258 ± 65	– (58)		

1% DMSO/RPMI[c]	620 ± 54	Vehicle		
Amoxicillin[d]			7	16

−: inhibited; *Acetylcholinesterase inhibitory activity. **Anti-inflammatory activity with measurement of TNF-α (in pg/mL) when co-cultured with the test compounds and their % inhibition. ***Antimicrobial activity against *H. pylori* showing both minimum inhibition zone (MIZ) and minimum inhibitory concentrations (MIC). Compounds were tested against seven bacterial and one fungal strain and the result shows only the compound-susceptible strain. [a]Reference drug for acetylcholinesterase activity. [b]Reference drug for anti-inflammatory activity. [c] Vehicle control for anti-inflammatory assay. [d]Reference drug for antimicrobial activity

Corydalis species have been widely studied for their anti-inflammatory isoquinoline alkaloids and many of them, especially tetrahydropalmatine (isolated from *C. yanhusuo*), pseudocoptisine (isolated from *C. turtschaninovii*) and ochotensimine (isolated from *C. impatiens*), significantly inhibited the production of IL-6, IL-8, TNF-α and also other mediators that are involved in the inflammation process [3]. In inflammatory diseases, the pro-inflammatory cytokine, TNF-α, plays a central role in inflammation and autoimmune diseases. Thus, the pro-inflammatory cytokine (TNF-α) in bacterial lipopolysaccharide (LPS) activated THP-1 cells are commonly used for evaluating the anti-inflammatory effects of various plant materials [6,14]. Using this model, we have earlier investigated the anti-inflammatory activity of the crude methanol extract of *C. dubia* and showed that it significantly inhibited the production of pro-inflammatory cytokine –TNF-α with 62% inhibition, which appeared better than the positive control (dexamethasone) with 58% inhibition [8]. Encouraged by this, we have further investigated the anti-inflammatory activity of the two major compounds – scoulerine (**1**) and cheilanthifoline (**2**) – isolated from *C. dubia*. Both compounds exhibited only weak anti-inflammatory activity (Table 1). Of the two compounds tested, cheilanthifoline (**2**) inhibited TNF-α cytokine production in LPS activated THP-1 cells by 13% in comparison with the solvent control (1% DMSO/RPMI). Since compounds **1** and 2 did not show significant anti-TNF-α activities, we believe that there may be some minor chemical components in the crude extract, which were lost during our isolation process.

Corydalis species and their isoquinoline alkaloids have also demonstrated antimicrobial properties including antiviral, antifungal and antibacterial [3,15]. Li and co-workers studied *in vitro* anti-*Helicobacter pylori* activity of 30 Chinese herbal medicines used for treating gastric ulcers in traditional medicine of China and discovered that the ethanolic extract of *C. yanhusuo* showed moderate antibacterial activity with a MIC value of 60 mg/mL [3]. Antimicrobial activity is assessed by both agar well diffusion (based on the zone of inhibition) and broth dilution (minimum inhibition concentration) methods. According to the broth dilution screening method for antimicrobial activity of higher plants, a prominent antibacterial effect worthy of further investigation is said to be as low as 1/32 dilutions-based inhibitory activities [16-17]. Rojas *et al*. [18] considered plant extracts and derivatives as bioactive antimicrobials if the MIZ is ≥3 mm well diameter. Svetaz *et al.* [19], Mohamad *et al*. [20] and Geyid *et al.* [21] reported plant extracts and their constituents as antimicrobial agents if their minimum inhibition concentrations (MIC) were in the range of 1000 - 4000 µg/mL. This suggests that a researcher can set the criteria of antimicrobial activity depending upon their interest of microbial strains under investigation. In this study, we have interpreted the results of test compounds as inhibitory only if the MIZ was ≥5 mm well diameter or if their MIC was ≤300 µg/mL. Values below 5 mm well diameter or above 300 µg/mL were considered either as inactive or not showing inhibition. We first assessed the inhibition zones of scoulerine (**1**) and cheilanthifoline (**2**) against seven bacterial strains (*Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, methicillin resistant *S. aureus* (MRSA), *S. epidermidis*, *Vibrio cholerae*, and *Helicobacter pylori*) and one yeast strain (*Candida albicans*), and then finally determined the MIC of the compounds that showed MIZs of ≥5 mm well diameter. While cheilanthifoline (**2**) showed the higher MIZ of 6 mm against only *B. subtilis* and *H. pylori*, this activity did not translate proportionately when its MIC was determined (MIC = 2000 µg/mL, not active). On the other hand, scoulerine (**1**) showed a MIZ of 5 mm against the *H. pylori* strain (Table 1) and demonstrated a better MIC value (250 µg/mL) in comparison with cheilanthifoline (**2**). The crude extracts of *C. dubia*, from which compounds **1** and **2** were isolated, exhibited weak antibacterial activity against *S. aureus*, MRSA, *B. subtilis* and *S. epidermidis* with MIZs of 5–6 mm [4]. Both alkaloids were inactive against the bacterial and yeast strains tested here.

Corydalis species are known AChEIs [3,22]. Acetylcholinesterase (AChE) is responsible for the termination of nerve impulse transmission at the cholinergic synapses by rapid hydrolysis of acetylcholine (ACh), which is a neurotransmitter linked to Alzheimer's disease (AD). AD affects more than 20 million people every year and is increasing [23]. Drug discovery within the AD field has for the last 20 years been rather amyloidcentric and only recently have other mechanisms and targets for screening been considered, including ones connected to inflammation, oxidative stress and the mechanistic intervention approach by targeting neurotransmitter dysfunction. Inhibitors of AChE currently form the basis of drugs for the management of AD, including the alkaloid – galanthamine, which was isolated from *Galanthus woronowii* [24]. AChE inhibitors (AChEI) increase the availability of acetylcholine in central cholinergic synapses and improve memory and cognitive deficits of the patients by diminishing the breakdown of acetylcholine at the synaptic site in the brain [3, 22-23]. Various assay protocols to screen for AChEI exist [23,25] and the choice of an appropriate screening assay is crucial. Di Giovanni *et al*. [26] have compared two colorimetric screening assays frequently used to detect acetylcholinesterase inhibitory activity (Marston *et al*. [27] and Ellman *et al*. [28]) involving a test set of 138 compounds. They obtained similar results and concluded that both AChE inhibitory screening assays are suitable for the generation of new hits. In this study, we used the method described by Marston *et al*. [27] and interpreted the result as described in Sastruruji *et al*. [29]. The TLC plate loaded with varying concentrations of the test compounds was sprayed with AChE enzyme stock solution, which was prepared from acetylcholinesterase of electric eels (EC 3.1.1.7, 906 U/mg). Structures of the human recombinant and eel enzymes are very similar [23], but they cannot be considered as identical, nor will inhibitors as a rule give identical results. Of the two alkaloids tested, scoulerine (**1**) exhibited a highly significant AChE inhibitory activity at a 1 ng concentration with a minimum inhibitory requirement (MIR) value of 0.0015 nmol, which was two-fold better than the reference drug, galanthamine (1 ng) with a MIR value of 0.003 nmol (Table 1). Cahlikova *et al*. [30] earlier reported that this same compound exhibited anti-AChE activity with an IC_{50} value of 245 µM against human AChE using the method described by Ellman *et al*. [28]. Obviously, the results of these two studies (ours versus Cahlikova *et al*. [30]) cannot be compared since different materials and methods were used, including target enzymes, colorimetric methods and the plant species. However, both these studies validated that scoulerine (**1**), though obtained from two different plant species (*Eschscholzia californica* and *C. dubia*), is a good AChEI. Cheilanthifoline (**2**) exhibited moderate AChE inhibitory activity with a MIR value of 0.31 nmol.

In conclusion, our study showed that scoulerine (**1**) and cheilanthifoline (**2**) have broad-spectrum biological activities, such

as anti-inflammatory, antibacterial and anti-AChE. These findings validated the traditional uses of *C. dubia* in Bhutanese medicine. We also confirmed that scoulerine (**1**) is a good AChEI. This alkaloid is a simple, non-toxic, small molecule [7] and has the physio-chemical properties of drug-like compounds that meet the criteria of the Lipinski rule of 5 [31]. Considering this, scoulerine (**1**) is worthy of further exploration including lead optimization, structure-activity-relationship studies, analogue development, pharmacodynamics and *in vivo* animal studies.

Experimental

Plant materials and preparation of compounds for biological testing: *Corydalia dubia* Prain was collected, processed and chemically assessed, as previously described by us [7]. Briefly, alkaloids **1** and **2** (along with 6 other alkaloids, as described in Wangchuk *et al.* **[**7]) were obtained through acid-base fractionation of extracts focusing on the alkaloids and then repeatedly purified by column and preparative thin layer chromatography (PTLC). Final separation of the basic fraction using PTLC plates with a mobile phase - $CHCl_3$/ethyl acetate (55:45 v/v) yielded scoulerine (**1**) and the other fraction furnished cheilanthifoline (**2**) (PTLC plate in a mobile phase of $CHCl_3$/ethyl acetate/NH_4OH) (55:45:4 drops). Stock solutions of these compounds were prepared in accordance to the requirements of different assay protocols.

Anti-AChE activity assay: The AChE inhibitory activity of scoulerine (**1**) and cheilanthifoline (**2**) were determined using the rapid TLC bioautographic method described by Marston *et al.* [27] and Sastraruji *et al*. [29]. The TLC plates were washed with acetone, dried, and the test compounds applied to the plates in varying quantities. The plate was sprayed with AChE enzyme stock solution, which was prepared from acetylcholinesterase of electric eels (EC 3.1.1.7, 906 U/mg). The plates were incubated at 37°C for 20 min and then sprayed with freshly prepared indicator solution (made from 1-naphthyl acetate and Fast Blue B salt to give the plate a purple coloration after 1-2 min); the compounds that inhibited AChE exhibited white spot on the plates. Galanthamine was used as a reference compound. The tests were performed in triplicate.

Anti-inflammatory assay: This assay was performed as previously described [6,8,14]. Briefly, THP-1 human monocytic cell lines (ATCC, TIB 202) were maintained in RPMI 1640 (Gibco-Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibco-Invitrogen, USA) in tissue culture flasks (Corning, USA) and were incubated (37**°**C in humidified 5% CO_2 incubator). For bioassays, cells were counted with a hemocytometer under an inverted microscope (Nikon TMS No. 300679: Nikon, Japan) and diluted to desired cell densities. THP-1 cells (200 µL) were seeded in 96-well tissue culture plates (Corning, USA) at a final density of 2.5 x10^5 cells/mL. Cells were incubated with the compounds alone or in combination with purified LPS (100 ng/mL) from *Escherichia coli* serotype O127 : B8 (Sigma, USA) for 3.5 h. Supernatants were collected from individual wells by centrifugation and assayed for TNF-α. The cell viability of compounds in THP-1 cells was determined by the Trypan-blue (Gibco-Invitrogen, USA) stain exclusion assay. Cells were incubated with varying concentration of compounds (0, 1.95, 3.9, 7.8, 15.6 and 31.25 µg/mL). The cell suspension was mixed with Trypan Blue, which stained dead cells. The percentage of cell viability was calculated by the formula: % Cell viability = 100 × (1−(dead cells/total cells)). TNF-α production in THP-1 cell culture supernatants was measured using cytokine-specific sandwich quantitative enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions (R & D Systems, USA). Recombinant human TNF-α was used as standard at concentrations of 15.6, 31.5, 62.5, 125, 250, 500, and 1000 pg/mL. Absorbance was measured at 450 nm using a BioTek® Synergy™ HT (Multi-Detection Microplate Reader, USA). Cytokine concentration in each well was quantified from a standard curve and expressed as pg/mL of culture medium. A commercial anti-inflammatory drug, dexamethasone (10 µg/mL), (Atlantic LabsComp. Ltd., Thailand) was used as a positive control. The experiments were performed 3 times in triplicate. The percentage of TNF-α inhibition was calculated by the formula: % TNF-α inhibition = 100 × ((observed/baseline) − 1). Where observed = secreted TNF-α of experiment (pg/mL) and baseline = secreted TNF-α of DMSO (pg/mL).

Antimicrobial test methods: The antimicrobial assay was performed as previously described [4,6,32]. Briefly, 7 bacterial strains (*Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), methicillin resistant *S. aureus* (MRSA), (DMST 20651), *S. epidermidis* (ATCC 12228), *Vibrio cholerae* (DMST 2873), and *Helicobacter pylori* (H40)) and on YEAST strain {*Candida albicans* (ATCC 10231)} were used for the study. *C. albicans* was grown on Sabouraud Dextrose Agar (SDA) (Becton Dickinson, USA) and incubated (25 °C, 24-48 h). *H. pylori* was grown on Brain Heart Infusion (BHI) agar (Becton Dickinson, USA) supplemented with horse serum (10%) (GIBCO Invitrogen, England) and incubated (at 37°C for 3 days) under micro-aerobic conditions (5% O_2, 10% CO_2, 85% N_2) generated by using a micro-aerobic GasPak (Mitsubishi, Japan) in an anaerobic jar (Mitsubishi, Japan). Other bacterial strains were grown on Mueller Hinton Agar (MHA) (Becton Dickinson, USA) under aerobic conditions (37°C, 24 h). The inocula of test organisms were prepared in appropriate broth by adjusting to the McFarland standard. A modified agar well diffusion method (AWD) [33-34] was used for determining the minimum inhibition zones (MIZ in mm diameters) for each compound. The MIC was examined by the broth microdilution method, as described by Wayne [35]. A serial two-fold dilution of each compound prepared in 96-well microtiter plates (Corning, USA) (stock concentration of 3 mg/mL) was used for measuring the minimum inhibitory concentration (MIC). Amphotericin B (Sigma-Aldrich,USA), vancomycin (Edicin, Slovenia) and amoxicillin **(**Government Pharmaceutical Organization**,** Thailand) were used as reference drugs against the yeast, MRSA and other bacterial strains, respectively. DMSO and distilled water were used as controls. The experiments were performed 3 times in duplicate.

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